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14. ABSTRACT
Using Affymetrix gene expression profiling on 2 independent sets of human breast tumor samples with known ER, PR, and Her2-neu status, we were able to molecularly profile breast tumors and identify a list of kinases that were differentially expressed in ER-negative tumors. Supervised clustering analysis based on ER status was performed and a gene expression profile was generated using 779 known and putative human kinases. Analysis in two independent sets of tumor samples identified 70 and 84 differentially expressed kinases, respectively (2.3 fold higher in ER-negative tumors, p-value <.05). The intersection of these lists contained 37 kinases. Additionally, unsupervised clustering analysis in both sets seemed to identify kinases that defined ER-negative, Her2/neu positive tumors as well as ER-negative, Her2/neu negative tumors. Overexpresssion of kinases was confirned and siRNA knockdown of kinases identified in the microarray analysis identifies several kinases that are critical for ER-negative, but not ER-positive breast cancer cell growth.

15. SUBJECT TERMS
estrogen receptor-negative breast cancer, genomic profiling, kinases

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INTRODUCTION

Although ER-positive breast cancers account for 60-70% of breast cancers, 30% of breast cancers are ER-negative and poorly responsive to traditional therapies *(1).* Selective estrogen receptor modulators (SERMs) (such as tamoxifen and raloxifene) and aromatase inhibitors reduce ER-positive breast cancer recurrence by approximately 50% (*2,3*). These agents, however, are not effective in treating or preventing ER-negative breast cancer. Currently, chemotherapy is used to treat ER-negative tumors *(4).* Such therapy is generally toxic and is not specifically targeted to ER-negative breast cancer, but instead non-specifically kills rapidly dividing cells. The only targeted therapy shown to be effective for a subset of ER-negative breast cancer is herceptin, a monoclonal antibody that only targets those tumors that overexpress the Her2 receptor *(4).* This information taken collectively demonstrates that to make additional advances in preventing and treating breast cancer, effective agents for ER-negative breast cancer must be developed.

It is evident that multiple signal transduction pathways play crucial roles in breast cancer development. The growth signal sensed by the cell is conveyed to the nucleus through interactions of proteins in series, each one activating another, through signal transduction pathways. Once the signal is received in the nucleus, transcription factors activate genes important for cell growth and survival. As was noted earlier, many of these pathways are understood in ER-positive cancers and have been the targets of small molecule inhibitors that can interrupt this mitogenic signaling, preventing and treating these cancers. Currently the mechanisms governing ER-negative breast cancer cell growth are unknown. It is clear that estrogen signaling is not the pathway that governs the mitogenic pathway, but despite the best efforts of numerous groups, the identification of pathways critical for ER-negative growth remains elusive. Recent advances in molecular biology have allowed for breakthroughs in the search for these growth pathways. Genome-wide expression arrays have allowed researchers to probe expression profiles in all different tissue types, including normal and malignant tissue *(5,6,7).* These studies, along with subsequent validation of their results, have led to advances in understanding breast cancer and have led to better tools for the clinician in evaluating patients with breast cancer. It is now possible to profile a tumor molecularly and determine what types of therapies will be most effective *(8,9,10).* Despite this increasing knowledge, however, it is clear that much work remains to be done.

BODY

1) Research Training Environment

The Breast Center at Baylor College of Medicine (BCM) provides a unique training environment with multiple opportunities for me to grow as a young research scientist. In the past year, I have taken full advantage of these opportunities as outlined:

- completed and received an "A" letter grade in the Molecular Carcinogenesis course taught here at BCM
- completed a course in Translational Breast Cancer Research, which is taught by faculty members of the Breast Center
- successfully completed my qualifying exam
- presented data in poster format at the 2007 Keystone Symposium on Molecular Targets in Cancer, Whistler, British Columbia, the graduate student symposium at BCM, the Medical Scientist Training Program Annual Retreat at South Shore Harbor, and the Annual Dan L. Duncan Cancer Center Symposium at Baylor College of Medicine
- Was awarded second place for poster presentation at the Medical Scientist Training Program Annual Retreat at South Shore Harbor
- Investigator in a recently funded Phase II clinical trial entitled, "A Biologic Correlative Study of Dasatinib, a Multi-Targeted Tyrosine Kinase, in "Triple-Negative" Breast Cancer Patients"
- Accepted and was awarded a travel grant to attend the Cold Spring Harbor Course entitled, "Integrated Data Analysis for High Throughput Biology"

2) Research Project

Specific Aim 1: Identify novel targets for the treatment of ER-negative breast cancer using genomic analysis:

1.1) Identify the kinases and phosphatases that are differentially expressed in human ER-negative vs. ER-positive breast tumors using RNA affymetrix microarray chips.

1.2) Validate that the genes are differentially expressed in a second set of human ER-negative breast cancers using quantitative RT-PCR analysis.

1.3) For selected identified kinases or phosphatases determine whether these proteins are differentially expressed in ER-negative vs. ER-positive tumors

In the studies we want to identify signaling molecules critical for the growth of ER-negative breast cancer. To begin to identify these mitogenic regulators, we first employed Affymetrix RNA expression profiling technologies. We have already completed preliminary microarray experiments on several independent sets of human breast tumor samples with known ER, PR, and Her-2/neu status. The arrays were completed using the Affymetrix HG U133A chip containing 22,000 human genes. For data analysis and clustering purposes, we chose to focus specifically on the human kinome, which is comprised of 779 known and putative human kinase genes. This profiling analysis served a two-fold purpose. First, supervised clustering analysis was able to identify those kinases that can differentiate ER-negative from ER-positive breast cancers and was able to identify those kinases that were more highly expressed in ER-negative human breast tumors (**figure 1**). Second, unsupervised clustering analysis on only ER-negative breast tumor samples allowed us to identify

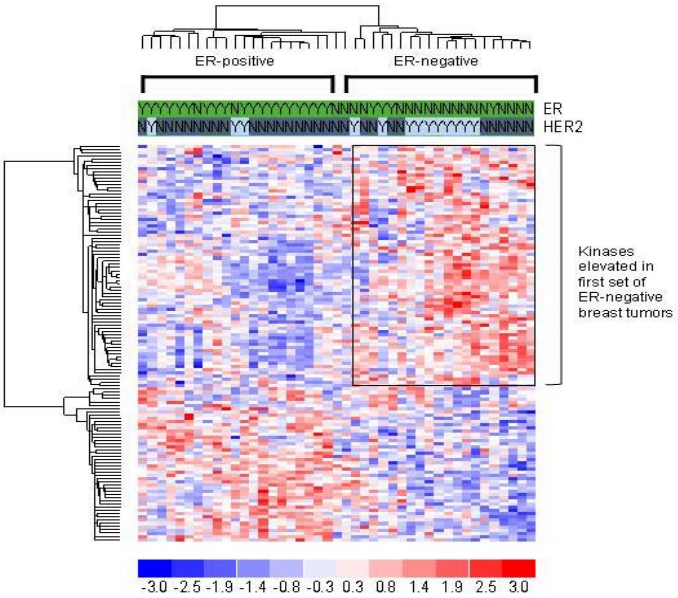

Figure 1- Supervised clustering analysis of kinases that distinguish ER-positive from ER-negative human breast tumors. Analysis reveals a cluster of kinases that are more highly expressed in ER-negative human breast tumors

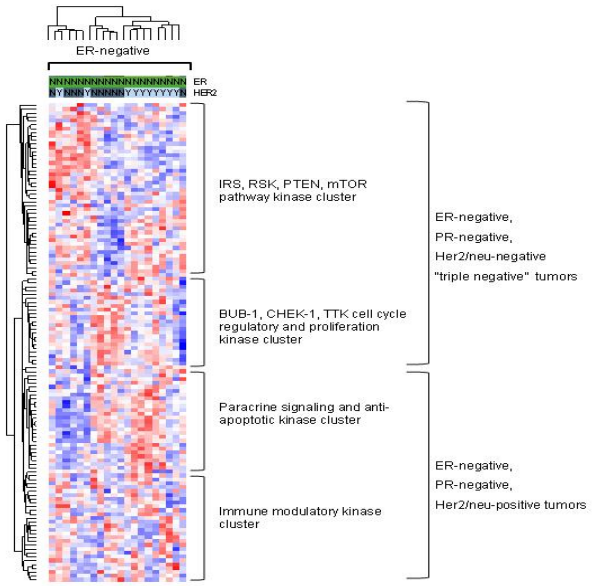

Figure 2- Unsupervised clustering analysis of kinases only in ER-negative tumors reveals 4 distinct subsets of ER-negative breast cancer. 2 of these subsets define ER-neg., PR-neg., Her2/neu-neg. "triple negative" tumors. The other 2 subsets define ER-neg, PR-neg, Her2/neu-positive tumors. These subsets are defined by kinases that control cell cycle, mitogenesis, apoptosis, and metabolism.

clusters of kinases that define biologically distinct subsets of ER-negative breast cancer (**figure 2**). Most broadly, this unsupervised kinase clustering was able to distinguish ER-negative, Her2-negative breast cancers from their ER-negative, Her2-positive counterparts. More specifically, however, further subsets within these two groups were identified, and they were unique as to the types of kinases overexpressed in these subsets. These results are intriguing, not only because triple negative tumors are characterized by being clinically more aggressive and less amenable to treatment, but because it is the first time to our knowledge that biologically distinct subtypes of ER-negative cancers can be identified based on their kinase expression profile. Because this analysis was done on multiple independent sets of human breast tumors, we chose to focus on those kinases

Metabolism	Anti-Apoptosis
phosphoglycerate kinase 1	vaccinia related kinase
phosphofructokinase, platelet	death-associated protein kinase 1
phosphoribosyl pyrophosphate synthetase 1	MALT lymphoma translocation gene 1
pyridoxal (pyridoxine, vitamin B6) kinase	mitogen-activated protein kinase 1 (ERK2)
selenophosphate synthetase 1	pim-1 oncogene /// pim-1 oncogene
uridine-cytidine kinase 2	serine/threonine kinase 17b
UDP-glucose pyrophosphorylase 2	v-raf-1 murine leukemia viral oncogene homolog
adenylate kinase 2	
Cell cycle/DNA damage checkpoint	**Positive regulation of cell proliferation**
BUB1	v-yes-1 Yamaguchi sarcoma viral oncogene
CHK1 checkpoint homolog	abl-interactor 1
TTK protein kinase	chemokine (C-X-C motif) ligand 10
serum/glucocorticoid regulated kinase	EPH receptor B4
SFRS protein kinase 1	serine/threonine kinase 38 like (NDR2)
PTK7 protein tyrosine kinase 7	v-raf-1 murine leukemia viral oncogene homolog
Immunoregulatory	**Positive regulation of transcription**
interleukin-1 receptor-associated kinase 1	interleukin-1 receptor-associated kinase 1
lymphocyte-specific protein tyrosine kinase	
toll-like receptor 1	
Receptor mediated endocytosis	**Unclassified**
PI4-kinase	LIM domain kinase 2
mindbomb homolog 1 (14-3-3)	maternal embryonic leucine zipper kinase
	protein kinase, X-linked
	RYK receptor-like tyrosine kinase
Cell-cell signaling	
chemokine (C-C motif) ligand 4	
myelin protein zero-like 1	

Figure 3- List of the 37 kinases identified by Affymetrix RNA expression profiling as being more highly expressed in ER-negative breast tumors. Gene ontology analysis shows that these kinases have varying biological functions, but most regulate growth and mediate apoptosis

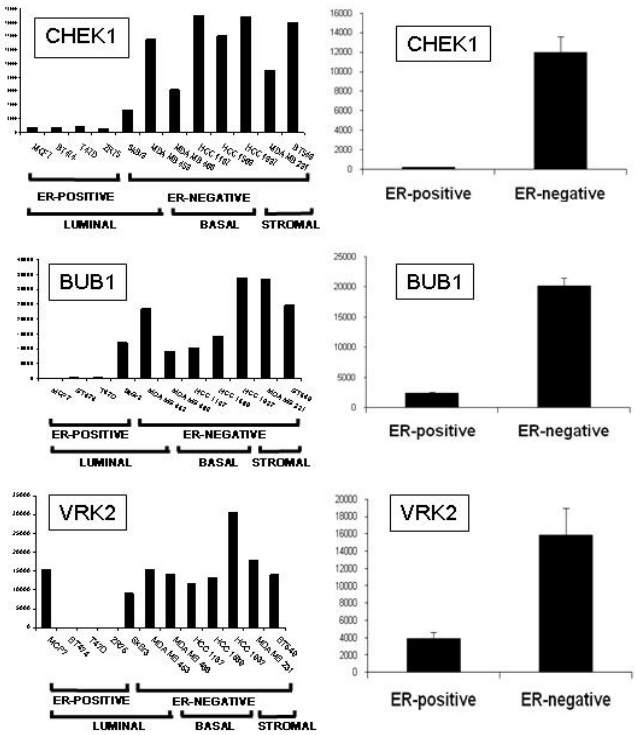

Figure 4- RNA expression of kinases in a panel of breast cancer cell lines chosen to accurately recapitulate the variety of human breast cancers. All kinases identified in the array show higher expression in ER-negative breast cancer cell lines by Q-PCR

that showed overlap between the tumor sets analyzed. Overlap analysis revealed 37 kinases expressed at least 2.3 fold higher in ER-negative breast cancer with a p-value of <.05 in all sets of human breast tumor samples (**figure 3**). Utilizing this list of 37 kinases, we have begun to validate these hits by evaluating mRNA levels in using quantitative RT-PCR (Q-PCR) assays. Analysis of kinase expression in a number of breast cancer cell lines (both ER-negative and ER-positive) has shown that all of the kinase hits identified through Affymetrix technology can be validated using Q-PCR (**figure 4**). In every case, the kinases identified as being more highly expressed in ER-negative breast cancers as compared to ER-positive breast cancers have validated in breast cancer cell lines. We are currently extending these Q-PCR validation assays into a completely independent set of human breast tumors from which RNA has been isolated. To further identify those kinases which may be of particular biological importance, we have classified these kinases according to their known biological functions. Gene ontology analysis of the identified kinases shows that they are enriched for genes that are important in positive regulation of cell proliferation, metabolism, and anti-apoptosis. Using this list of identified kinases, we wanted to understand what, if any, role these kinases play in mitogenesis. To begin to understand what role the identified kinases play in this process, siRNAs were designed against the kinases, used to knockdown target kinase expression, and proliferation studies were performed (as described in Aim 3).

Specific Aim 2: Identify novel targets for the treatment of ER-negative breast cancer using proteomic analysis:

2.1) Make protein lysates from the 110 human tumor samples (both ER-positive and ER-negative) and use these lysates in a reverse phase tissue lysate array. This is a quantitative automated proteomics assaying system that determines the expression level and activation status of signaling proteins. Using this array technology I will identify those signaling molecules that are differentially expressed between ER-negative and ER-positive tumors. Furthermore, I will assay for activation status of the molecule utilizing phospho-specific antibodies.

2.2) Validate that selected identified proteins or phosphoproteins are differentially expressed in a second set of human ER-negative breast tumors using western blotting.

We are currently working with our collaborators to run the proteomic arrays and the results should begin to be available in the next 3 weeks. We have already made all of the protein lysates from the 110 human tunor samples, quantitated their concentration, and prepared them for spotting on the high-density arrayer. After analyzing the results, we will proceed to validation and gene ontology characterization.

Specific Aim 3: Determine whether inhibition of the identified RNA and protein targets suppresses ER-negative breast cancer growth *in vitro* and *in vivo*.

3.1) For *in vitro* studies I will determine whether inhibition of signaling molecule function using siRNA knockdown inhibits ER-negative breast cancer cell growth. For these experiments I will use ER-negative cell lines selected to accurately represent *in vivo* breast cancers. These cells will be transfected with siRNA designed against signaling molecules identified in **Aim 1** and **2.** I will then use MTT, soft agar growth, and invasion assays to determine whether specific gene knockdown inhibits growth or invasion.

3.2) Use existing small molecule inhibitors of the identified signaling molecules to block the activity of these proteins and assay for growth suppression.

3.3) For *in vivo* studies, I will determine whether stably transfected shRNA or small molecule inhibitors can suppress the growth of breast tumors when xenografted into nude mice.

To evaluate the role of kinase function in both ER-negative and ER-positive breast cancer growht, cell lines representing these two types of cancers (MDA-MB-468 and MDA-MB-231; and MCF-7 and T47D cells, respectively) were used in the siRNA experiments. Several of the kinases evaluated to date have significant growth inhibitory phenotypes when knocked down in ER-negative breast cancer cell lines, while they show no growth inhibitory effects in ER-positive cell lines (**figure 5**). Also, progress is currently being made in evaluating the effect of all 37 kinases indentified in the intersection in Aim 1 in further siRNA knockdown experiments. These results will be available in the next 3 months. Additionally, we are beginning to plan *in vivo* mouse xenograft experiments that will be conducted in the next 6 months. Finally, based on the results of these studies, we are proceeding with a phase II clinical trial that has been funded and IRB approved to evaluate whether the multi-kinase inhibitor dasatinib can regress advanced triple negative breast tumors in women who have failed all other therapies.

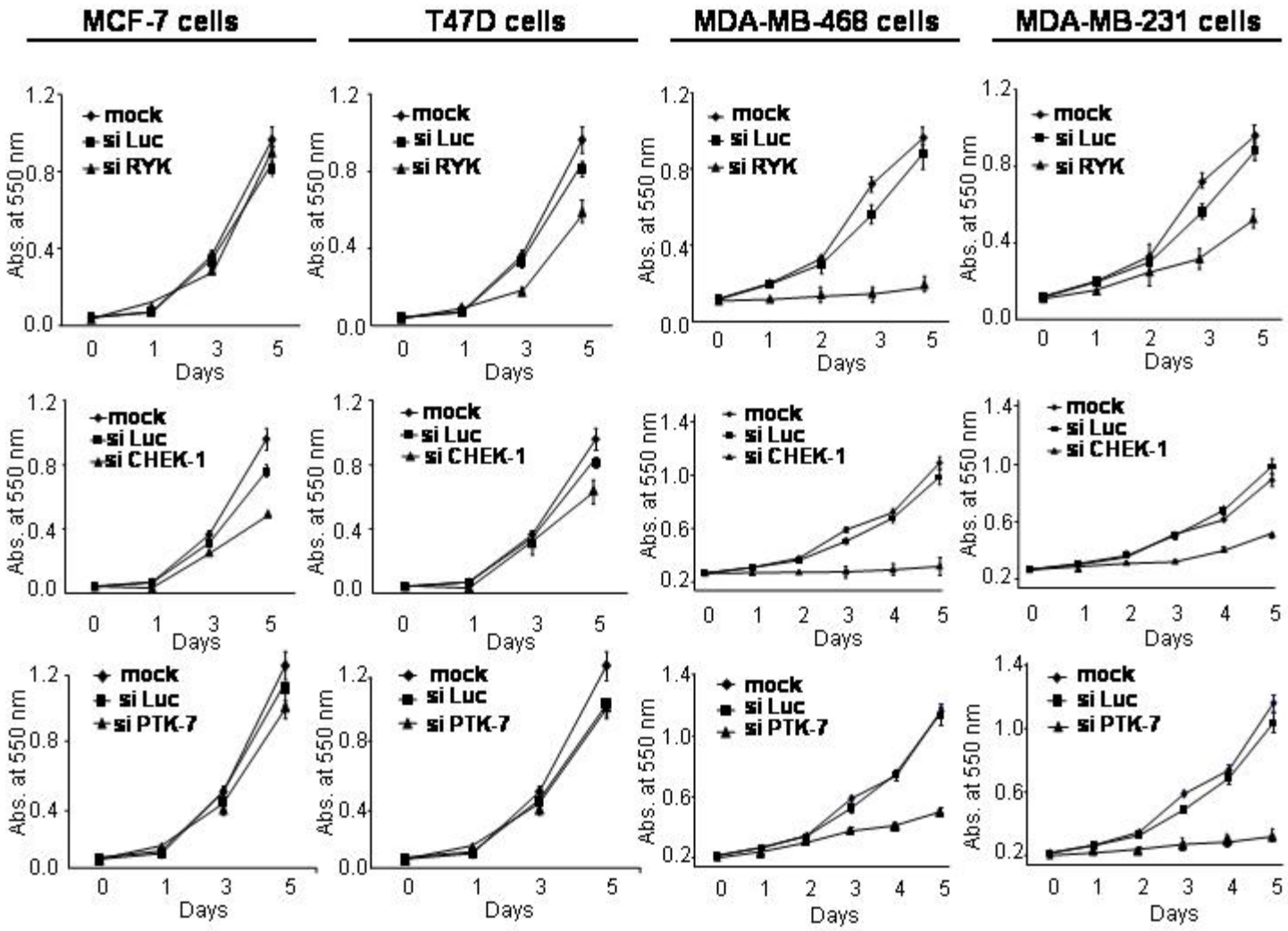

Figure 5- siRNA knockdown of genes identified in specific aim 1 shows that these kinases are critical for the growth of ER-negative, but not ER-positive, breast cancer cell lines.

KEY RESEARCH ACCOMPLISHMENTS

- Gene expression microarray analysis is a robust means of identifying kinases upregulated in ER-negative breast tumors.
- Unsupervised clustering analysis identifies 4 distinct subsets of ER-negative breast cancer
- Identified kinases can be validated using Q-PCR and western blot analysis (data not shown) in both breast cancer cell lines and human breast tumors
- *In silico* promoter analysis identifies E2F4 as a regulator of expression in one of the 4 identified subsets of ER-negative breast cancer
- Inhibition of the several identified kinases using siRNA inhibits ER-negative, but not ER-positive, breast cancer cell growth *in vitro*

- Based on the kinase targets identified in this research, the multi-kinase inhibitor dasatinib, which targets many of the kinases identified in this analysis, is being taken into a phase II clinical trial that we are heading.

REPORTABLE OUTCOMES

- see attached publication currently being submitted to Cancer Cell

CONCLUSION

We conclude that gene expression microarray analysis is a robust means of identifying kinases upregulated in ER-negative breast tumors and have identified specific kinases that are highly expressed in ER-negative breast cancers as compared to ER-positive breast cancers. Furthermore, quantitative RT-PCR analysis confirms that these kinases are also elevated in ER-negative breast cancer cell lines and a third independent set of human breast tumors. Finally, knockdown of gene expression using siRNA technology inhibits proliferation of ER-negative cell lines with no effect on ER-positive breast cancer cell growth, suggesting that signaling through pathways involving these kinases may be necessary for ER-negative breast cancer growth. These kinases may serve as druggable targets for the treatment of ER-negative breast cancer. Indeed, a phase II clinical trial has been recently approved and is accruing patients based on the results of these experiments. It is our hope that these and future experiments will lead to more targeted treatments for breast cancer.

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APPENDIX

Corey W. Speers

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EDUCATION

B.S.	Summa Cum Laude, Brigham Young University, 2002 Provo, UT **Medical Microbiology**
M.D./Ph.D.	Baylor College of Medicine, 2003-present

HONORS and AWARDS

2002	1st place for a presentation entitled, "The effects of diallyl sulfide, diallyl disulfide, garlic, and quercetin on benzo[a]pyrene induced DNA damage in HepG2 cells as measured by the comet assay" at the Annual Intermountain Meeting of the American Society of Microbiologists in March of 2001.
2002	Recipient of the Teagle Scholarship for outstanding academic achievement.
1999-2002	Recipient of an academic scholarship from Brigham Young University
2002	Graduated Summa Cum Laude from Brigham Young University
2005	Finalist for the BRASS scholarship
2006	Recipient, DOD Pre-doctoral fellowship for grant entitled, "Novel strategies for the treatment of estrogen receptor-negative breast cancer"
2007	Accepted to Cold Spring Harbor Course entitled, "Integrated Data Analysis for High Throughput Biology"
2007	Awarded travel grant to attend Cold Spring Harbor Course entitled "Integrated Data Analysis for High Throughput Biology"

RESEARCH EXPERIENCE

2000-2002	Independent researcher in Dr. Kim O'Neill's cancer research laboratory evaluating the anti-genotoxic effects of flavonoids
2002	SMART program participant in 2002 at Baylor College of Medicine working with Dr. Powel Brown, MD, PhD investigating the chemopreventative effects of ZD1839 (Iressa) on human breast cancer
2002-2003	Laboratory Technician in Dr. Powel Brown's laboratory at Baylor College of Medicine continuing evaluation of ZD1839
2005-2007	Graduate student, Dr. Powel Brown's laboratory, evaluating the growth regulation of estrogen receptor-negative breast cancer

PUBLICATIONS

Chunhua Lu, Corey Speers, Yun Zhang, Xiaochun Xu, Jamal Hill, Emily Steinbis, Joseph Celestino, Qiang Shen, Heetae Kim, Susan Hilsenbeck, Syed K. Mohsin, Alan Wakeling, C. Kent Osborne, and Powel H. Brown **Effect of Epidermal Growth Factor Receptor Inhibitor on Development of Estrogen Receptor–Negative Mammary Tumors** J Natl Cancer Inst 2003; 95: 1825-1833

Speers C, Ahlstrom J., Murray BK, O'Neil KL. **The effects of diallyl sulfide and diallyl disulfide on benzo[a]pyrene induced DNA damage in HepG2 cells as measured by the comet assay**, Proceedings of the American Association for Cancer Research, March 2002

Speers C, Ahlstrom J., Murray BK, O'Neil KL**. Inhibition of benzo[a]pyrene induced DNA damage in Hep G2 cells by various organosulfur compounds as measured by the comet assay**, American Society of Microbiologists News, May 2002

Speers C, Ahlstrom J., Murray BK, O'Neil KL. **Anti-genotoxic effects of Garlic extract and Quercetin as Measured by the Comet Assay**, The Journal of the Intermountain Branch of the American Society of Microbiologists, March 2002

Speers C, Ahlstrom J., Murray BK, O'Neil KL. **Antimutagenic Effects of Diallyl Disulfide and Allyl Disulfide on Benzo (a) pyrene induced DNA Damage in Hep G2 Cells as measured by the Single Cell Gel Electrophoresis and Glutathione S-Transferase Assays**, ORCA News, August 2002

PRESENTATIONS

2002	Poster presentation, 93rd Annual Meeting of the American Association for Cancer Research in San Francisco.

2002	Poster presentation, Annual Meeting of the American Society of Microbiologists
2002	Talk, Annual Meeting of the American Society of Microbiologists, Intermountain Meeting.
2007	Poster Presentation at Keystone Symposium on Molecular Targets in Cancer, Whistler, British Columbia
2007	Poster presentation, Molecular and Cellular Biology Student Symposium

TEACHING EXPERIENCE

1999-2000	German Instructor, Brigham Young University
2000	Techniques in Molecular Biology Laboratory, Brigham Young University
2000-2002	Biology Student Instructor, Brigham Young University

GRANTS and FELLOWSHIPS

2001-2002	Recipient of two ORCA research scholarships to fund independent cancer research
2002	Recipient of the Cancer Research Center's Research Fellowship Grant
2002	Recipient of the ASM Travel Grant to attend the Annual Meeting of the American Society of Microbiologists.
2003-present	Teagle Foundation Scholarship for academic excellence
2006-present	DOD Pre-doctoral fellowship, Three year award
2007	Investigator, Phase II clinical trial entitled, "A Biologic Correlative Study of Dasatanib, a Multi-Targeted Tyrosine Kinase, in "Triple-Negative" Breast Cancer Patients"

EXTRACURRICULAR ACTIVITIES

2006-present	Member, Student Advisory Committee, Department of Molecular and Cellular Biology
2006-present	Member, Student Operating Committee, MSTP program
2007	Organizer of National Youth Leadership Forum on Medicine to Baylor College of Medicine

PROFESSIONAL ORGANIZATIONS

- American Medical Student Association
- Texas Medical Student Association
- American Association for Cancer Research
- American Society of Microbiologists
- Delta Phi Alpha, the National German Honor Society
- National Society of Collegiate Scholars

NEW FOR 2006-2007

2007	**Poster Presentation at Keystone Symposium on Molecular Targets in Cancer, Whistler, British Columbia**
2007	**Poster presentation, Molecular and Cellular Biology Student Symposium**
2007	**Accepted to Cold Spring Harbor Course entitled, "Integrated Data Analysis for High Throughput Biology"**
2007	**Awarded travel grant to attend Cold Spring Harbor Course entitled "Integrated Data Analysis for High Throughput Biology"**
2007	**Investigator, Phase II clinical trial funded entitled, "A Biologic Correlative Study of Dasatanib, a Multi-Targeted Tyrosine Kinase, in "Triple-Negative" Breast Cancer Patients**
2007	**Organizer of National Youth Leadership Forum on Medicine to Baylor College of Medicine**
2006-present	**Member, Student Advisory Committee, Department of Molecular and Cellular Biology**
2006-present	**Member, Student Operating Committee, MSTP program**

Identification of Novel Kinase Targets for the Treatment of Estrogen Receptor-Negative Breast Cancer

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Summary:

Previous gene expression profiling studies in breast cancer have focused on the entire genome for identification of genes differentially expressed between estrogen receptor (ER)-positive and ER-negative cancers. Here we report a distinct kinase gene expression profile that identifies ER-negative breast tumors and which also is able to subset ER-negative breast tumors into 4 distinct subtypes. Further, we show that the kinases identified in this manner are validated as being more highly expressed in ER-negative cancers using a panel of breast cancer cell lines. Kinase expression knock-down studies show that several of these kinases are essential for the growth of ER-negative, but not ER-positive, breast cancer cell lines. Finally, survival analysis shows that overexpression of the identified kinases confers poor overall and relapse-free survival. This study identifies a list of kinases that are prognostic and may serve as druggable targets for the treatment of ER-negative breast cancer.

Significance:

Breast cancer is the number one cause of cancer-related deaths in women worldwide. It is estimated that in 2007, over 40,000 women will die from this disease in the United States alone. About 60-70% of breast cancers are ER-positive; these tumors are effectively treated by selective estrogen receptor modulators (SERMs) or aromatase inhibitors. ER-negative cancers are clinically more aggressive and currently have limited effective, targeted therapies. Here we apply gene expression profiling to identify a set of kinases that are differentially expressed in ER-negative breast tumors. Furthermore, we show that these kinases are essentially for mitogenesis in ER-negative breast cancer cell lines. This study provides rationale for the development of additional kinase inhibitors that may be effective in the targeted treatment of ER-negative breast cancer, particularly the aggressive “triple negative” breast cancer.

Introduction:

The genomic era has produced an exponential increase in our understanding of cancer biology and has greatly accelerated cancer drug development. With the advent and implementation of microarray expression profiling, it is now possible to evaluate gene expression in tumors on a genome-wide basis. Gene expression microarray analysis is now being extensively used to subtype cancers, predict prognosis and disease free survival, and determine optimal treatment (1-5).

ER-positive breast cancers account for 60-70% of breast cancers, but there remains 30% of breast cancers that are ER-negative and poorly responsive to traditional therapies (6). Selective estrogen receptor modulators (SERMs), such as tamoxifen and raloxifene, and aromatase inhibitors have been shown to reduce ER-positive breast cancer recurrence by approximately 50% (7, 8). These agents, however, are not effective in treating ER-negative breast cancer. Currently, chemotherapy is used to treat ER-negative tumors (9). Such therapy is generally toxic and is not specifically targeted to ER-negative breast cancer, instead only non-specifically killing rapidly dividing cells.

The goal of current breast cancer treatment research has been the identification of targets that are unique to cancer cells and that can be effectively targeted to effect only the cancerous cells with no effect on normal tissue. While this is an elusive goal, there are several examples of effective targeted therapies, including the development of monoclonal antibodies like trastuzumab (targeting the Her2/neu receptor) and bevacizumab (targeting vascular epithelial growth factor) which have been shown to be effective in treating breast cancer (10, 11). Other success stories include the development of small molecule tyrosine kinase inhibitors like gefitinib and erlotinib (both of which target the epidermal growth factor receptor), and lapatinib (a dual kinase inhibitor targeting both the epidermal growth factor receptor and the Her2/neu receptor) (12-15). The most effective treatment for ER-negative breast cancer is trastuzumab, a monoclonal antibody developed to treat Her2/neu overexpressing tumors. While effective, these ER-negative, Her2 positive tumors constitute only 10-15% of all tumors. Recent efforts to develop targeted therapies for the remaining ER-negative breast cancer have used expression microarray technology to the identify molecules that play a role in breast cancer development and progression. Subsequent validation of these findings, along with the development of specific targeted inhibitors of these molecules, will certainly broaden treatment options and improve patient survival

The purpose of this study was to identify the kinases that are over-expressed in ER-negative breast cancer that may serve as druggable targets for the treatment of ER-negative breast cancer and in particular “triple negative” breast cancer. We have used genomic profiling data to evaluate the expression of kinases and have identified a set of kinases which are critical for the growth of ER-negative cells. These results demonstrate that ER-negative breast cancer can be sub-divided into 4 separate subgroups, each of

which over-express a specific set of kinases. These kinases represent promising targets for the treatment of ER-negative breast cancers.

Results:

To identify kinases that are differentially expressed in ER-negative breast cancers, we designed a study intended to compare kinase expression levels in ER-positive and ER-negative human breast tumor samples. A summary of the study design is outlined in **figure 1**.

Patient Population:

A total of 102 patients with invasive breast cancer were recruited through IRB-approved, neoadjuvant studies to investigate gene expression in human tumors before and after drug treatment. These 102 patients were divided into two sets for the purposes of this study. Diagnostic biopsies were taken before initiation of any treatment and were used in this study. Because the patients did not receive systemic adjuvant or neoadjuvant therapy prior to biopsy, the results from the gene expression analysis represent basal gene expression in these breast cancers. For these gene expression profiling experiments, the tumor biopsies were divided into two groups (referred to as set 1 and set 2). The first set (see **table 1**) included 43 breast tumor samples, 23 of which were ER-positive and 20 ER-negative by IHC-staining. The tumors were all stage III or IV from pre- and post-menopausal women, with all tumors showing >30% cellularity. The PR and *Her2/neu* status for these tumors was determined by IHC-staining. The second set of tumors contained 59 breast tumor samples, 35 of which were ER-positive and 24 ER-negative (see **table 1**). As with the set 1 tumors, all were stage III and IV tumors from pre- and post-menopausal women Again, PR and *Her2/neu* status was known and all tumors had >30% cellularity. Clinical and demographic features of the two sets are summarized in **table 1**. There were no differences in age and BMI between the two tumor sets. The set 1 tumors did tend to have a higher percentage of premenopausal women and palpable nodes at baseline, but otherwise the two sets were comparable.

Affymetrix gene expression profiling identifies kinases overexpressed in human ER-negative breast tumors.

To identify signaling molecules that are differentially expressed in ER-negative breast cancers, we performed Affymetrix gene expression profiling to compare human ER-negative and ER-positive breast tumors. A subsequent data analysis and clustering was limited to the known kinome with interrogation of the 779 known and putative human kinases listed in **supplementary table S1**. We first performed D-chip analysis to identify those kinases that were differentially expressed in ER-positive and ER-negative breast

tumors. This analysis revealed a significant difference ($P < 0.05$) in the expression of 124 kinases between ER-negative and positive tumors. To visualize the clustering of the ER-positive and ER-negative tumors, supervised clustering analysis was done using only those kinases identified as being differentially expressed between the two groups (**figure 2**). Supervised clustering showed that these 124 kinases were able to segregate ER-positive and ER-negative tumors and that the majority of the *Her2/neu* positive tumors were ER-negative (as expected). Upon further analysis, 70 of these 124 differentially expressed kinases were expressed at least 2.3 fold higher in the ER-negative breast tumors as compared to ER-positive tumors. These 70 kinases were selected for further study.

Unsupervised clustering analysis reveals four distinct subtypes of ER-negative breast cancer

We next determined whether this list of 70 kinases overexpressed in ER-negative breast cancers could sub-cluster the ER-negative tumors in an unbiased manner. We performed unsupervised clustering analysis using only the ER-negative breast cancer samples and found that these tumors clustered broadly into 4 distinct subtypes of ER-negative breast cancers (**figure 3**). Two of these clusters defined distinct subgroups of ER-negative, PR-negative, *Her2/neu*-negative ("triple negative") breast cancer. The other two cluster groups defined distinct subgroups of ER-negative, PR-negative, *Her2/neu*-positive breast tumors. Upon further inspection of these four subsets of tumors, there is one subset of "triple negative" tumors (subgroup 1) defined by kinases in the mTOR pathway, including IRS-1, RSK, and S6 kinases. The other "triple negative" tumor subset (subgroup 2) is defined by kinases such as CHEK-1, BUB-1, TTK, and PTK7, that are involved in cell cycle checkpoint control and mitogenesis and has been called the "proliferation" cluster. Of the two other ER-negative, *Her2/neu* positive clusters, one (subgroup 3) was defined by kinases that govern paracrine growth signaling and anti-apoptosis (with the overexpression of v-Raf1, PTK7, and myelin protein zero-like 1 kinases). The fourth subset (subgroup 4) is defined by kinases that are involved in modulating the immune system (chemokine ligand 10, interleukin 1 receptor associated kinase 1, lymphocyte specific protein tyrosine kinase, toll-like receptor 1, and chemokine ligand 4). We have defined this subgroup as the "immunomodulatory" subset of ER-negative tumors.

A second, independent set of human breast tumors identifies 4 similar subsets of ER-negative breast cancer.

We next used the set 2 tumors to both validate kinases identified in the set 1 analysis and discover additional kinases that distinguish ER-negative from ER-positive tumors. We analyzed Affymetrix gene expression data from the 59 set 2 human breast tumors as described for the set 1 tumors. As with the first set of tumors, supervised clustering analysis was done using the list of 779 kinases to determine those that could discriminate between ER-negative and ER-positive tumors. In this analysis, we identified 237

kinases as being significantly different ($P < 0.05$) between ER-negative and ER-positive tumors (**supplementary figure 1**). While there was an increased number of kinases whose expression was found to be significantly different in ER-negative breast tumors as compared to ER-positive tumors, all 124 of the kinases identified using our set 1 tumors were included in this larger list of 237 kinases. The clustering of these set 2 kinases was very similar to that seen in set 1 tumors. In an attempt to again identify biologically distinct subsets of ER-negative breast tumors, unsupervised clustering analysis of only the ER-negative breast tumor samples from set 2 was performed. This analysis again identified 4 clusters that defined biologically distinct ER-negative breast cancers (**supplementary figure 2**). As with set 1, there were 2 subsets of tumors defined by high expression of kinases in the ER-negative, PR-negative, *Her2/neu*-negative, "triple negative" tumors, and 2 subsets of tumors that expressed specific kinases in the ER-negative, PR-negative, *Her2/neu*-positive tumors. We again found subgroups of tumors characterized by high expression of mTOR pathway (subgroup 1), proliferation (subgroup 2), paracrine growth stimulating (subgroup 3), and immunomodulatory kinases (subgroup 4). These results, taken together, serve as a validation of the set 1 analysis and underscore the utility of Affymetrix gene expression profiling as a means of identifying biologically distinct subsets of breast cancer. More importantly, these results indicate that ER-negative tumors are not homogenous in their kinase expression and that tumors can be sub-divided based on the levels of their kinase expression.

To narrow our candidate kinase list for further investigation, we chose to include only those kinases that were expressed at least 2.3 fold higher in ER-negative breast tumors as compared to ER-positive with a p-value <.05. By applying this criteria to both the set 1 and set 2 kinase lists, we were able to identify 70 and 84 kinases, respectively (**figure 4**). The intersection of the set 1 and set 2 lists included 37 kinases (**table 2**). This list of 37 became our candidate kinase list for further validation and interrogation. Thus, the initial potentially targetable kinase list went from 779 known and putative kinases to 37 kinases which were overexpressed in two independent sets of ER-negative human breast tumors.

Gene Ontology analysis

To gain insight into the potential function of kinases highly expressed in ER-negative breast cancer, we performed gene ontology (GO) enrichment analysis using EASE software and found that several classes of biological function were highly enriched in our selected sets (**table 2**). This included enrichment for kinases involved in the regulation of metabolism (p-value $<10^{-14}$), cell cycle (p-value $<10^{-12}$) and DNA damage checkpoint control (p-value $<10^{-11}$), cell-to-cell signaling (p-value $<10^{-9}$), and apoptosis regulation (p-value $<10^{-9}$). Many of these kinases fell in linear pathways, for example TTK, CHEK-1, BUB-1 kinases, all of which play a role via sequential phosphorylation and activation in regulating G2/M transitioning and DNA damage checkpoint control pathways.

Differentially expressed kinases can be further validated using publicly available data sets.

To demonstrate that these kinases are indeed overexpressed in ER-negative tumors compared to ER-positive tumors, we evaluated individual kinase expression using publicly available expression array data sets. These datasets come from studies by Wang *et al*., Richardson *et al*., vant Veer *et al*., and vandeVijver *et al*. (3, 5, 16, 17). In each of these datasets our kinases validated as being significantly more highly expressed in ER-negative breast tumors as compared to ER-positive tumors (**supplementary figure 3**).

Kinase overexpression in ER-negative breast tumors can be validated in breast cancer cell lines using Q-RT-PCR.

We next confirmed that our kinase "hits" identified in human breast tumors were also overexpressed in ER-negative breast cancer cell lines. Twelve ER-positive or ER-negative breast cancer cell lines were chosen and the expression of the identified kinases was measured under basal growth conditions. Results show that all kinases evaluated so far (18 kinases) have statistically significant increases in expression in the panel of ER-negative breast cancer cell lines as compared to ER-positive cell lines. Representative results for several of these kinases are shown in **figure 5**.

Identified kinases accurately cluster human breast cancer cell lines into ER-positive or ER-negative clusters in an unsupervised manner.

To determine whether our panel of 37 kinases can accurately subgroup breast cancer cell lines we used available expression data from 51 breast cancer cell lines. Recent work by Neve *et al*. showed that the recurrent genomic and transcriptional characteristics of breast cancer cell lines mirror those of primary breast tumors (18). These investigators performed Affymetrix gene expression profiling on a set of 51 ER-positive or ER-negative breast cancer cell lines and used hierarchical clustering to show that the cell lines clustered into three main groups: basal A, basal B, and luminal (18). We used this expression information to select breast cancer cell lines to determine whether our list of 37 kinases subgrouped these cell lines into similar subgroups as seen in human tumors (mTOR, proliferation, paracrine, and immunomodulatory). When hierarchical cluster analysis was performed on the expression data from these 51 cell lines using only the list of 37 kinases identified by expression profiling, the cell lines were accurately clustered into ER-positive or ER-negative groups. Furthermore, these 37 kinases were also able to accurately subcluster the luminal, basal A, and basal B subtypes of breast cancer identified by Neve *et al*., in an unsupervised manner (**figure 6**). These results indicate that the expression profile of the identified kinases is able to accurately discriminate between ER-positive and ER-negative breast cancer cell lines and may serve as a reliable diagnostic tool to categorize human tumors in the future.

Knock-down of several identified kinases inhibits the growth of ER-negative (MDA-MB-468 and MDA-MB-231) but not ER-positive (MCF-7 and T47D) breast cancer cell lines.

We next performed siRNA knock-down studies to determine the effect of individual kinase knock-down on breast cancer cell proliferation. ER-positive (MCF-7 and T47D) cells and ER-negative (MDA-MB-468 and MDA-MB-231) cells were transfected with siRNAs for each of the 37 kinases identified in our screen. All siRNA constructs used in the study showed at least 70% knockdown of target kinase expression for 4 days after transfection. After 4 days, kinase expression slowly increased until returning to pre-transfected levels by day 8. Knock-down of 9 of the 20 kinases evaluated so far (EPHB4, LIMK2, PIM1, YES1, RYK, VRK2, PTK7, vRAF1, UCK2) had a significant growth inhibitory effect on ER-negative MDA-MB-468 and MDA-MB-231 cells but had no effect on ER-positive breast cancer cells. An additional 5 of 20 kinases **(**BUB1, CHEK1, IRAK1, CCL4, TTK**)** inhibited growth of all breast cancer cell lines. Knock-down of 5 of the 20 kinases **(**STK38L, DAPK1, SFRS1, PKXL, TLR1**)** had no effect on any breast cancer cell line growth, while knock-down of 1 of 20 kinases (MPZL1) had a significant growth stimulatory effect on all breast cancer cell lines examined. Growth curves from some of the knock-down experiments are shown in **figure 7**. Knock-down of many of the kinases in the "proliferation" cluster of ER-negative breast cancer had a profound inhibitory effect on ER-negative breast cancer cell growth but no effect on ER-positive breast cancer, while knock-down of certain kinases in the "immunomodulatory" cluster inhibited the growth of all breast cancer cell lines examined. These results indicate that many of the kinases found to be highly expressed in ER-negative breast cancers are critical for breast cancer cell growth.

Set of 37 kinases predicts poor patient outcome and relapse free survival

In an effort to determine whether our identified list of differentially expressed kinases provided prognostic import, we analyzed the survival data from the Wang data set (3, 17).

Discussion:

In this report we show that Affymetrix gene expression profiling of human breast tumors is able to identify kinases that are differentially expressed in ER-negative breast cancers and can identify kinases that are more highly expressed in ER-negative breast cancers as compared to ER-positive breast cancers. The intersection of these two analyses identified 37 kinases that are expressed at least 2.3 fold higher in ER-negative breast tumors (P-value <.05). Further analysis reveals that these ER-negative tumors are able to be subtyped into 4 distinct subgroups depending on the levels of their kinase expression. Analysis of publicly available breast tumor data sets shows that these kinases are indeed upregulated in ER-negative breast cancers, and quantitative RT-PCR analysis confirms that these kinases are also elevated in ER-negative breast cancer cell lines as compared to ER-positive cell lines. Application of our list of 37 kinases to a set of 51 breast cancer cell lines shows that we can accurately cluster these ER-negative cell lines into the luminal, basal A, or basal B subgroups (as defined by Neve *et al*. (18)). Knock-down of gene expression using siRNA inhibits proliferation of ER-negative cell lines, suggesting that signaling through pathways involving these kinases is necessary for ER-negative breast cancer growth. Finally, analysis of metastasis-free survival data shows that overexpression of the 37 identified kinases confers a poorer prognosis and may be useful in the evaluation of patients with ER-negative cancer. This study identifies novel targets for the treatment of ER-negative breast cancer, including the aggressive "triple negative" form of breast cancer.

This is the first report to show that ER-negative breast cancers can be subdivided into biologically distinct groups based on the level of their kinase expression. Our data indicate that ER-negative breast tumors can be subdivided into 4 distinct sub-groups. These four clusters are defined by the expression of kinases in the mTOR pathway, proliferative kinases, paracrine and apoptotic signaling kinases, and kinases involved in immunomodulation. Though much work remains to be done to determine the biological and clinical import of these 4 subgroups, the final "immunomodulatory" subgroup identified in this report has recently become of a focus of increasing scientific inquiry. The role of the immune system in cancer has historically been viewed rather myopically, with investigation into how the immune system itself responds to the "foreign" cancer as the primary focus. It is now being appreciated that the tumor itself may act autonomously to influence the stromal microenvironment and evade recognition by the immunosurveillance machinery. Goldberg-Bittman *et. al.* have evaluated the expression of CXCL10, a kinase identified as being differentially expressed in this study, in human breast adenocarcinoma cell lines (19). Recent work by Teschendorff *et al.* has also identified an immunomodulatory profile in ER-negative breast cancer which was shown to confer better prognosis (20). Other groups have published conflicting results on the impact of immunomodulatory genes and what, if any, role they play in the development and prognosis of breast cancer (21, 22). It will be interesting to determine whether modulation of intrinsic gene expression by the tumor is an important mechanism by which cancer cells can avoid immunosurveillance, including the proper controls meant to keep aberrant

growth in check (20, 21). Furthermore, it will be interesting to determine whether the 4 subtypes of ER-negative breast cancer have significant differences in overall survival, disease free survival, and metastasis.

Given the paucity of effective, targeted therapies for the treatment of ER-negative breast cancer, these studies provide a large number of promising new targets for the treatment of ER-negative breast cancer. ER-positive breast cancers are now routinely treated and cured using SERMs and aromatase inhibitors, and these cancers are now even prevented using such pharmacologic intervention. Recent studies have shown that intrinsic breast cancer subtypes differ depending on the ethnicity of the patient from whom the tumor is obtained. Carey *et al.* refined an IHC-based assay to categorize the prevalence of varying breast cancer subtypes in different populations (23). It was shown that the prevalence of the basal-like subtypes was strongly influenced by race and menopause status. The highest prevalence of basal-like tumors was noted in premenopausal African American breast cancer patients (23, 24). Basal-like tumors, which are almost uniformly ER-negative, PR-negative, and *Her2/neu* negative (so called "triple negative") are known for being clinically more aggressive, carry a higher proliferative capacity, occur at a younger age, are less amenable to chemotherapy, and carry a much poorer prognosis (24, 25). This work provides the rational for targeted therapy to treat this type of cancer more common among a traditionally underserved population.

Several groups have previously shown that breast cancer is heterogeneous in its gene expression patterns and that utilizing this expression information allows for the identification of subtypes of human breast cancer (18, 26-29). These subtypes have proven useful in the characterization of human tumors and expression data is now being used to predict response to various treatment modalities (1-4, 27, 30, 31). Additionally, clinicians now are able to use gene expression levels to guide treatment decisions in cases of ER-positive breast cancer (31). Despite these advances, our ability to identify patients with ER-negative breast cancer who require tailored therapy is much less developed. Identifying markers that not only categorize tumors, but are also themselves potential treatment targets, will usher in the age of truly personalized medicine. One can envision a time when each new work-up of cancer will include a genetic and expression profiling of one's tumor that will provide the clinician with a complete list of aberrances in the tumor. Each deletion, translocation, overexpression will be known, as well as the pathway or pathways that are involved. With this list in hand, coupled with the further expansion of targeted therapies, clinicians will be able to tailor a treatment regimen for the particular type of cancer the patient has. Individualized, targeted treatment will then no longer be something hoped for in the future, but a reality that will increase survival and bring us closer to a cure for this deadly disease.

Experimental Procedures:

Study Population and Design:

Get from Dr. Chang

Human Breast Tumors

All ER-negative and ER-positive tumors were collected in collaboration with Dr. Jenny Chang through IRB-approved, neoadjuvant studies to investigate gene expression changes that occur in human tumors after drug treatment. Diagnostic biopsies were taken first, then several (up to 6) additional cores were taken for biomarker studies. Breast biopsies were taken before treatment, placed in liquid nitrogen, and used to prepare RNA, DNA, and protein. Immunohistochemical (IHC) staining for ER and *Her2/neu* expression was done on these sets of tumor samples as previously described (32).

Affymetrix microarray experiments

Total RNA from these tumor samples was isolated using Qiagen's RNeasy kit, double-stranded cDNA synthesized, and reverse transcription carried out followed by biotin labeling. Additionally, about 250-fold linear amplification and phenol-chloroform cleanup was done as previously published. From each biopsy, 15 micrograms of biotin-labeled cRNA was hybridized onto an Affymetrix HGU133A GeneChipTM which comprise around 22,000 genes. The analytical approach used was similar to previously described methods *(11).* Raw un-normalized data were processed and analyzed by dChip *(12)* and classifiers were constructed with BRB Array Tools *(13).* Gene expression was estimated with dChip software *(14),* using the Perfect Match (PM) only model because this model-based approach allows new datasets to be normalized and expression to be computed without reanalyzing all previous datasets, thus mimicking the scenario of a diagnostic clinical test where new samples can be tested and compared to a standard. A list of differentially expressed genes was obtained with BRB Array Tools using the Randomized Variance Model (RVM) method, which is designed to improve variance estimates when samples sizes are small. The experiments were all done in the microarray core at Baylor College of Medicine. Additionally, statistical analysis was done in collaboration with the Breast Center's statistician, Dr. Anna Tsimelzon, with additional input from Dr. Susan Hilsenbeck.

Selection of Genes for further study

After completing all microarray experiments and doing statistical analysis, including generating gene clustergrams, a group of 37 overlapping kinases was selected from the two tumor sets for further study. These genes had a minimum of 2.3 fold higher expression in ER-negative vs. ER-positive tumors with a p value <.05 in both Set 1 and Set 2 tumors.

RNA isolation and Quantitative RT-PCR (Q-RT-PCR)

Total RNA was isolated using the RNeasy RNA isolation kit (QIAGEN). Quantitative RT-PCR assays of transcripts were carried out using gene-specific double fluorescence-labeled probes in an ABI PRISM 7500 Sequence Detector (Applied Biosystem). The PCR reaction mixture consisted of 300nM each of the forward and reverse primers, 100nM probe, 0.025 units/µl of Taq Polymerase (Invitrogen), 125µM each of dNTP, 5mM MgCl2, and 1X Taq Polymerase buffer. Cycling conditions were 95°C for 30 seconds, followed by 40 cycles at 95°C for 5 sec. and 60°C for 30 sec. 6-Carboxy fluorescein (FAM) was used as the 5' fluorescent reporter and black hole quencher (BHQ1) was used at the 3'end quencher. All reactions were performed using triplicate RNA samples. Standard curves for the quantification of each transcript were generated using the serially diluted solution of synthetic templates. Results were reported as average expression ± standard error of the mean.

Western blot analysis

For protein isolation from tissue culture cell lines, cells were washed once with ice-cold phosphate buffered saline (PBS) and lysed in protein lysis buffer consisting of 50mM HEPES pH7.5, 150mM NaCl, 1mM EDTA, 1% Triton X-100, 10% glycerol, 100mM NaF, Complete Mini protease inhibitors cocktail tablet (Roche), and phosphatase inhibitor cocktail I and II (Sigma-Aldrich). Protein concentration was determined using BCA Protein Assay Reagents (Pierce Biotechnology). An aliquot of total protein (20µg) was resolved by electrophoresis in 10% SDS-PAGE gel and transferred to a nitrocellulose membrane (Amersham Biosciences). The membrane was blocked with 5% nonfat milk in 10mM Tris-HCl, pH 7.4, 150mM NaCl, and 0.1% Tween 20 (TBST) overnight at 4°C. Thereafter, the membrane was incubated with primary antibody diluted in 1% nonfat milk/TBST overnight at 4°C or 2 h at room temperature, after which the membrane was washed in TBST 3 times for 10 minutes each. The membrane was then incubated with horseradish peroxidase-conjugated secondary antibody diluted in 1% milk/TBST at room temperature for 1 h. The membrane was washed 3 times in TBST for 10 min each. Antigen-antibody complexes were detected using the ECL or ECL Plus chemiluminescent system (Amersham Bioscience). Primary antibodies specific for kinases of interest were purchased from Cell Signaling or Novacastra. Antimouse and anti-rabbit secondary antibodies were obtained from Amersham Bioscience. Protein isolation from human tumor

samples was identical except that samples were first homogenized using a 7 mm generator and a rotator-stator homogenizer (ProScientific). Samples were homogenized in protein lysis buffer and all isolation was done on ice.

siRNA transfection

siRNAs for all kinases (see supplementary table) were purchased from Sigma Aldrich. siRNA transfection was performed using DharmaFECT^{TM} 1 (Dharmacon), according to the manufacture's instruction. MDA-MB-468, MDA-MB-231, T47D, and MCF-7 cells were plated in 100 mm dishes and grown to 60% confluence before being transfected with Dharmacon siRNA dilution buffer (mock-transfection), 20 ng of kinase specific siRNA constructs, or with scrambled siRNA or mock transfected as a control. 36 hours after transfection, cells were replated in 96 well plates at a density of 2000 cells per well. RNA and protein were also harvested at this time (as described previously), as well as on day 2 and day 4, to confirm sufficient knockdown of kinase expression by Q-RT-PCR and western blotting, respectively. After replating in 96 well plates, growth was measured by MTS assay every 2 days for a total of 5 days.

Cell proliferation assays

Cell growth was measured using the CellTiter 96^{TM} Aqueous Non-Radioactive Cell Proliferation assay (MTS assay, Promega) according to the manufacturer's instruction. Briefly, cells were plated in 96-well plates at 2000 cells per well. Every 24 hours a solution containing 20:1 ratio of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and PMS (phenazine methosulfate) was added to the cells. Plates were incubated at 37°C for 2 h and absorption at 550nm was determined. Each data point was performed in heptuplicate, and the results were reported as average absorption ± standard deviation.

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Figure 1- Overview of the analysis methods and project design

Table 1- Characteristics of patient tumors used in Set 1 and Set 2 analysis

Figure 2- A) Set 1 supervised hierarchical clustering analysis of kinases that distinguish ER-positive from ER-negative human breast tumors. Analysis of the first set of 43 tumors reveals a cluster of 124 kinases that are differentially expressed between ER-negative and ER-positive human breast tumors with a p-value <.05. B) Unsupervised hierarchical clustering analysis of kinases only in ER-negative tumors from Set 1 reveals 4 distinct subsets of ER-negative breast cancer. Two of these subsets define ER-neg., PR-neg., *Her2/neu*-neg. "triple negative" tumors. The other two subsets define ER-neg, PR-neg, *Her2/neu*-positive tumors. These subsets are defined by kinases that control cell cycle, mitogenesis, apoptosis, metabolism and immune modulation.

Figure 3- Venn diagram showing those kinases identified in the Set 1 and Set 2 analysis that were expressed at least 2.3 fold higher in ER-negative tumors over ER-positive tumors with a p-value <.05. 70 kinases met this criteria in set 1 and 84 kinases in set 2. The intersection of these sets included 37 kinases.

Table 2- List of the 37 kinases identified by Affymetrix RNA expression profiling as being at least 2.3 fold more highly expressed in ER-negative breast tumors with a p-value <.05. Gene ontology analysis shows that these kinases have varying biological functions, but most regulate growth and mediate apoptosis.

Figure 4- RNA expression of kinases in a panel of breast cancer cell lines chosen to accurately recapitulate the variety of human breast cancers. All kinases identified in the array show higher expression in ER-negative breast cancer cell lines by Q-RT-PCR. Data are represented as mean ± SEM.

Figure 5- List of kinases validates in an independent data set of human breast cancer cell lines. Publically available breast cancer cell line expression data was clustered in an unsupervised manner using only the kinase genes identified in our analysis. Unsupervised hierarchical clustering using only the 37 intersection kinases between set 1 and set 2 is still able to distinguish ER-positive and ER-negative tumors and largely identify the luminal, basal A, and basal B subtypes.

Figure 6- Loss of kinase expression inhibits growth of ER-negative breast cancer cells but not ER-positive breast cancer cells. (A) Knockdown of target kinase expression was achieved using siRNA against identified kinases. Knockdown was confirmed by Q-PCR at day 2 and day 5. (B) Kinase knockdown inhibited growth in the ER-negative breast cancer cell lines MDA-MB-468 and MDA-MB-231 but not in the ER-positive breast cancer cell lines MCF-7 and T47D. Similar results were seen with other kinases identified in the screen (data not shown). Data are represented as mean ± SD

Supplementary Figure 1- Set 2 supervised hierarchical clustering analysis of kinases that distinguish ER-positive from ER-negative human breast tumors. Analysis of this set

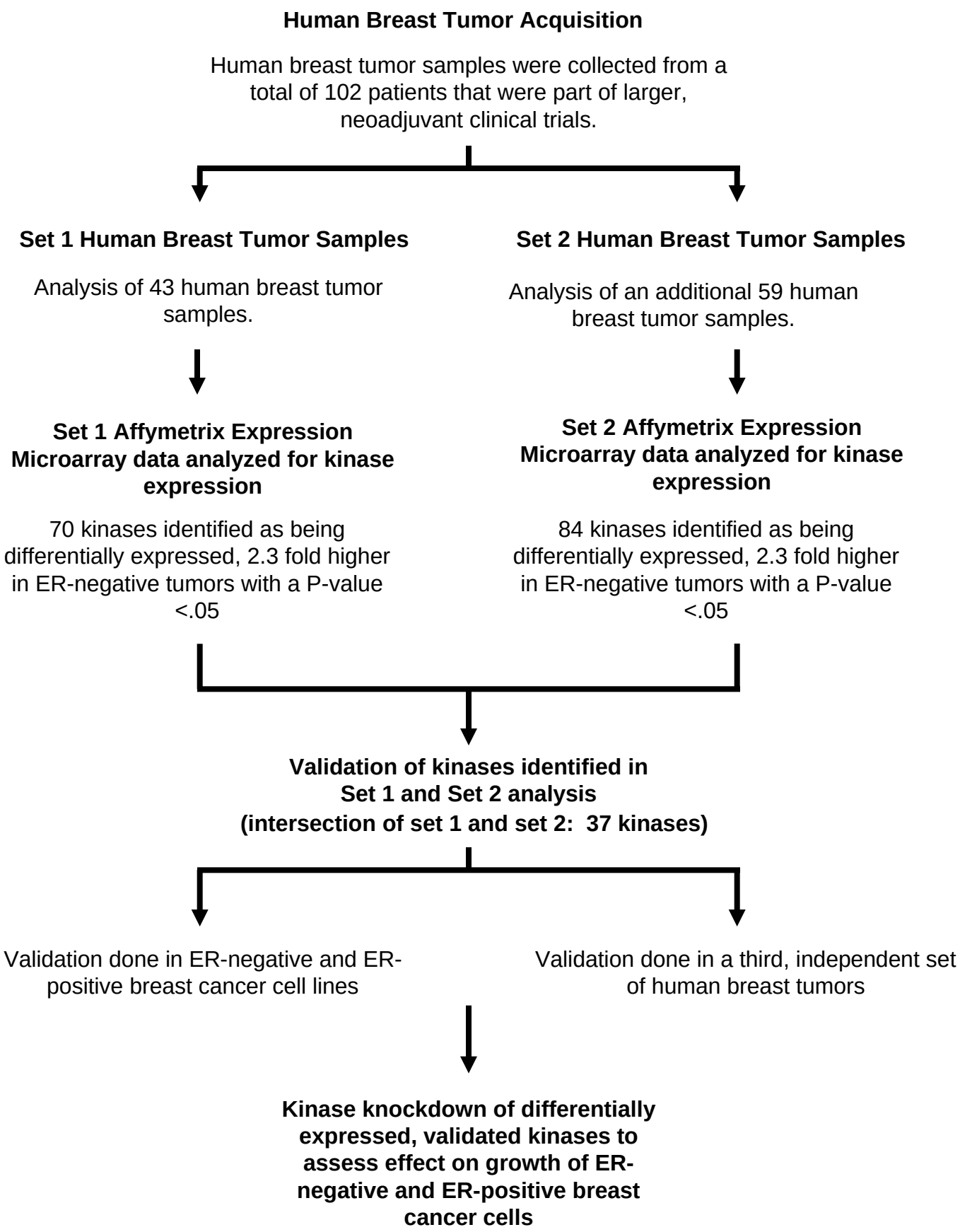
of tumors reveals a cluster of 237 kinases that are more highly expressed in ER-negative human breast tumors with a p-value <.05.

Supplementary Figure 2- Unsupervised hierarchical clustering analysis of kinases only in ER-negative tumors from set 2 again reveals 4 distinct subsets of ER-negative breast cancer. As with set 1 tumors, two of these subsets define ER-neg., PR-neg., *Her2/neu*-neg. "triple negative" tumors. The other two subsets define ER-neg, PR-neg, *Her2/neu*-positive tumors. Similar to the set 1 analysis, these subsets are defined by kinases that control cell cycle, mitogenesis, apoptosis, metabolism, and immune modulation.

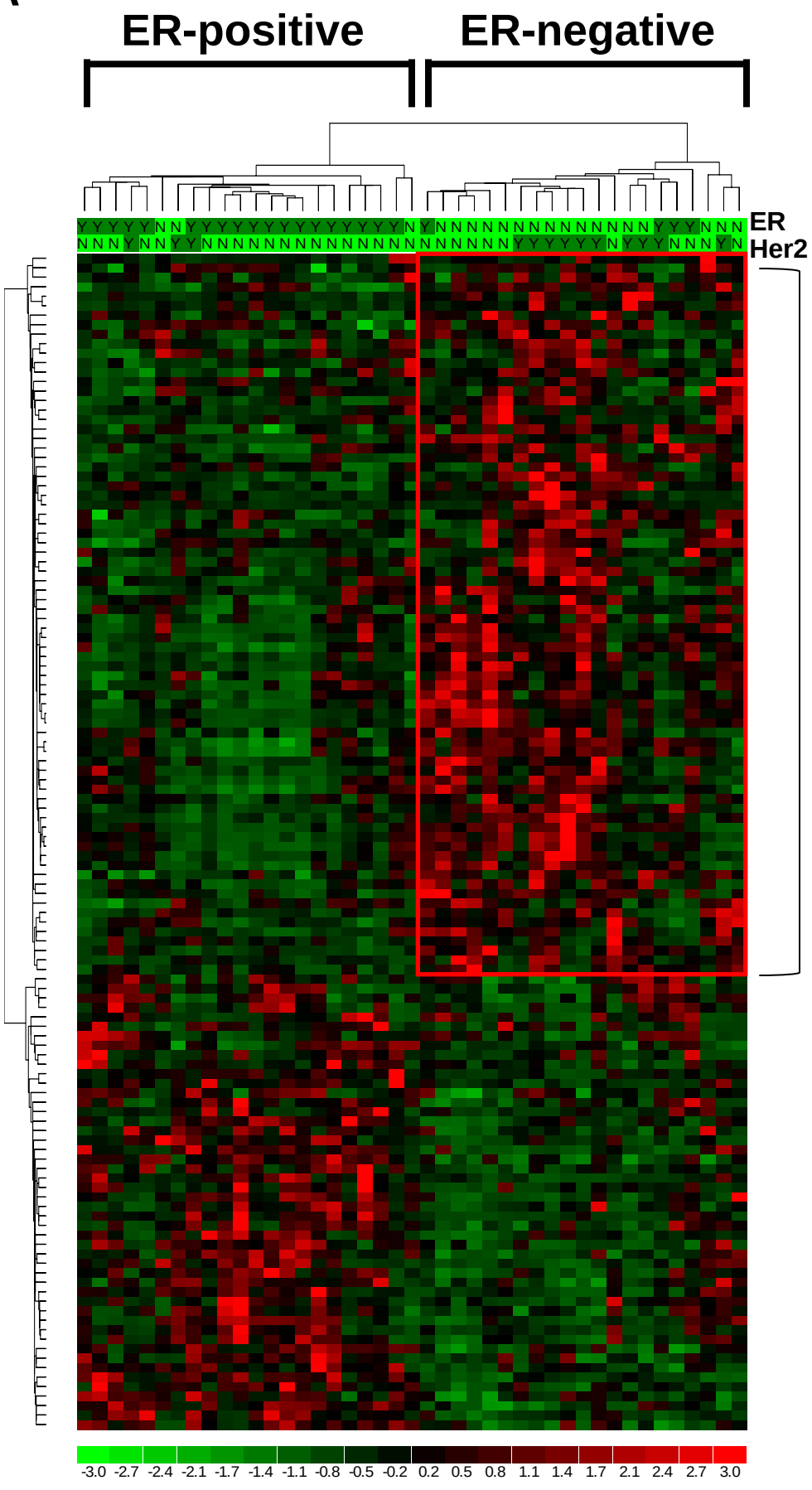
Supplementary Figure 3- RNA expression of kinases in a third, independent set of tumors. Kinase expression was evaluated by Q-PCR assay in 37 human breast tumor samples. Kinases identified as being overexpressed in ER-negative breast cancer by microarray analysis validated as being overexpressed in this tumor set as well. Data are represented as mean ± SEM.

Supplementary Table 1- characteristics of the three human tumor sample sets used in the study. The first two tumor sets were used in Affymetrix gene expression profiling. The third tumor set was used as an independent validation set for Q-RT-PCR analysis of kinase expression.

Set 1 Tumor Samples		Set 2 Tumor Samples	
Characteristic	**Set 1 N=43 (%)**	**Characteristic**	**Set 2 N=59 (%)**
Age		*Age*	
Mean	46.2	Mean	49.5
Range	(32-67)	Range	(32-72)
Race		*Race*	
Caucasian	12 (43%)	Caucasian	38 (64%)
Hispanic	0 (0%)	Hispanic	7 (12%)
African-American	15 (53%)	African-American	8 (14%)
Asian	1 (4%)	Asian	6 (10%)
Menopausal Status		*Menopausal Status*	
Pre	29 (69%)	Pre	20 (54%)
Post	13 (31%)	Post	17 (46%)
BMI		*BMI*	
Mean	31.5	Mean	28.8
Range	(16.1-48.3)	Range	(19.1-46.5)
Baseline Tumor Size, cm		*Baseline Tumor Size, cm*	
Mean	7.2	Mean	5.6
Range	(2.5-25.0)	Range	(3.0-17.0)
Palpable Nodes at Baseline		*Palpable Nodes at Baseline*	
Yes	15 (35%)	Yes	7 (12%)
No	28 (65%)	No	52 (88%)
ER		*ER*	
Positive	22 (51%)	Positive	35 (59%)
Negative	21 (49%)	Negative	24 (41%)
Unknown	0 (0%)	Unknown	0 (0%)
PR		*PR*	
Positive	9 (21%)	Positive	28 (48%)
Negative	19 (44%)	Negative	28 (48%)
Unknown	15 (35%)	Unknown	3 (4%)
HER2/Neu		*HER2/Neu*	
Positive	19 (44%)	Positive	8 (17%)
Negative	24 (56%)	Negative	34 (54%)
Unknown	0 (0%)	Unknown	17 (29%)

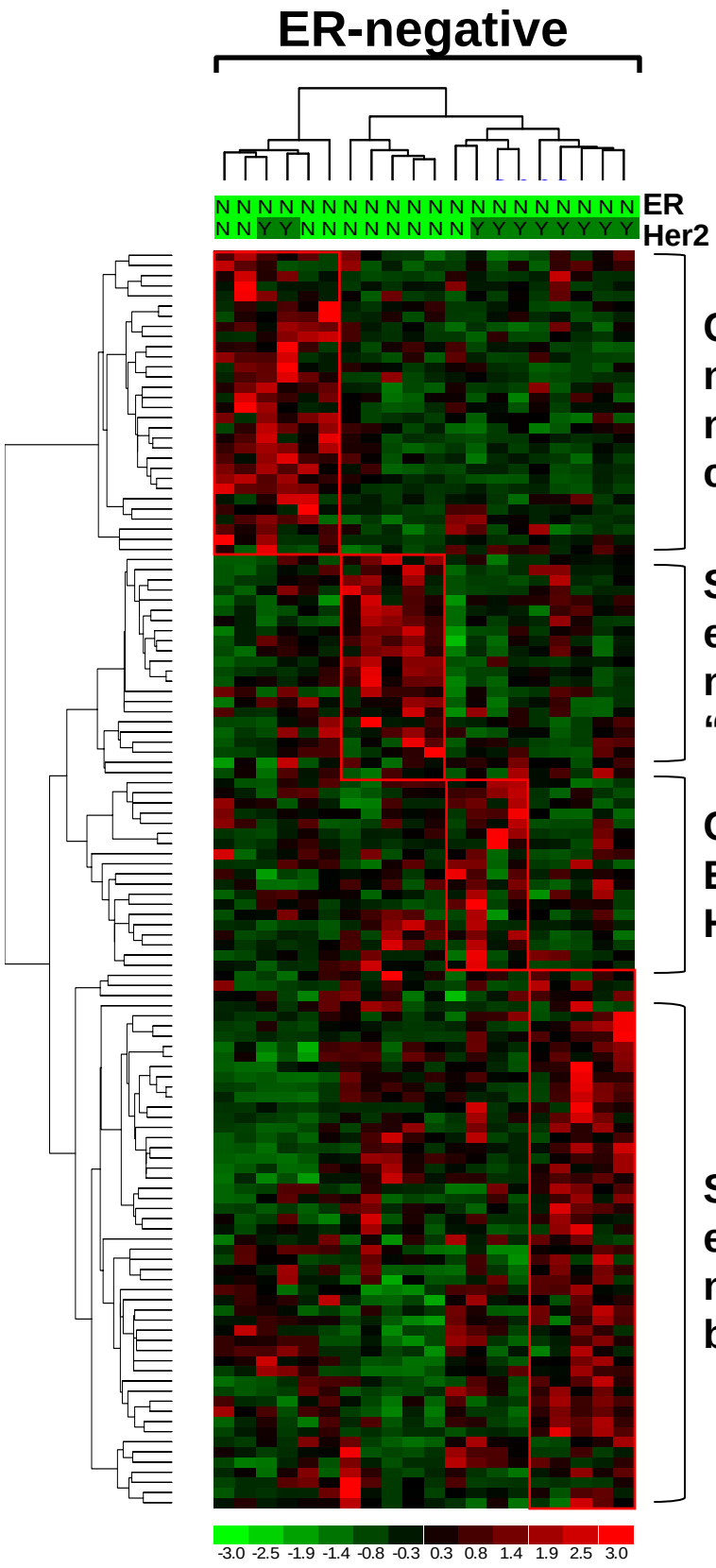

Human Breast Tumor Acquisition
Human breast tumor samples were collected from a total of 102 patients that were part of larger, neoadjuvant clinical trials.
Set 1 Human Breast Tumor Samples
Analysis of 43 human breast tumor samples.
Set 2 Human Breast Tumor Samples
Analysis of an additional 59 human breast tumor samples.
Set 1 Affymetrix Expression Microarray data analyzed for kinase expression
70 kinases identified as being differentially expressed, 2.3 fold higher in ER-negative tumors with a P-value <.05
Set 2 Affymetrix Expression Microarray data analyzed for kinase expression
84 kinases identified as being differentially expressed, 2.3 fold higher in ER-negative tumors with a P-value <.05
Validation of kinases identified in Set 1 and Set 2 analysis (intersection of set 1 and set 2: 37 kinases)
Validation done in ER-negative and ER-positive breast cancer cell lines
Validation done in a third, independent set of human breast tumors
Kinase knockdown of differentially expressed, validated kinases to assess effect on growth of ER-negative and ER-positive breast cancer cells

A



Kinases elevated in first set of ER-negative breast tumors

B



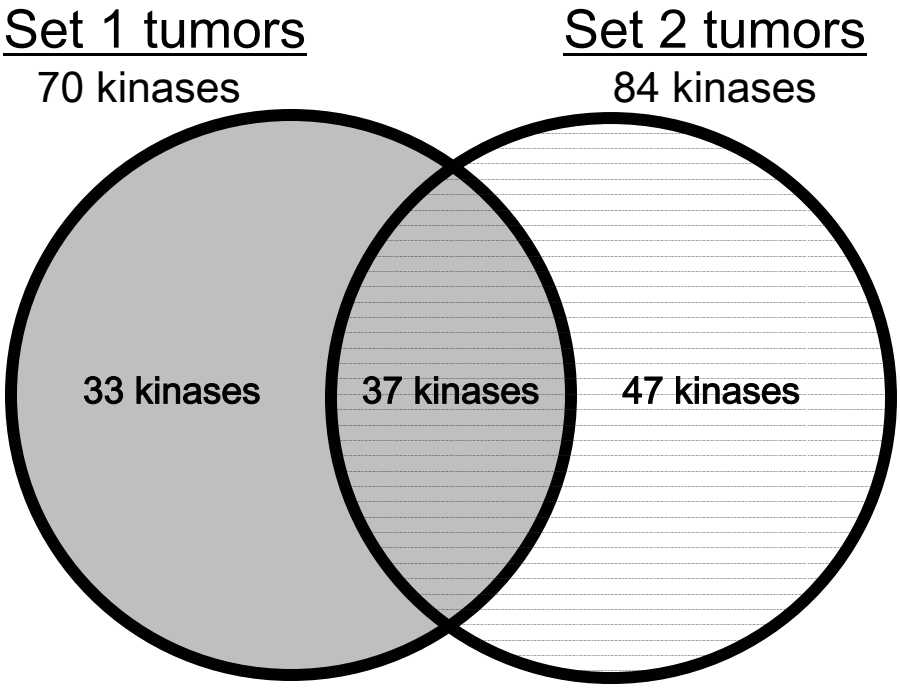
Cluster of kinases elevated in ER-negative, PR-negative, Her2/neu negative "triple negative" breast cancer

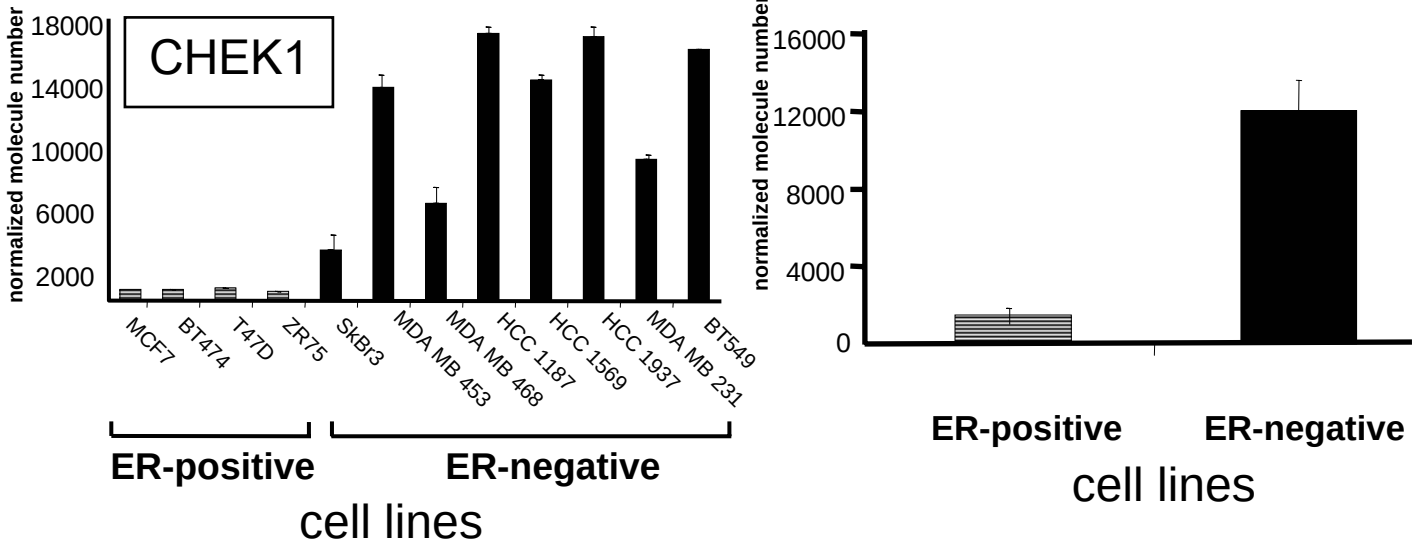
Second cluster of kinases elevated in ER-negative, PR-negative, Her2/neu negative "triple negative" breast cancer

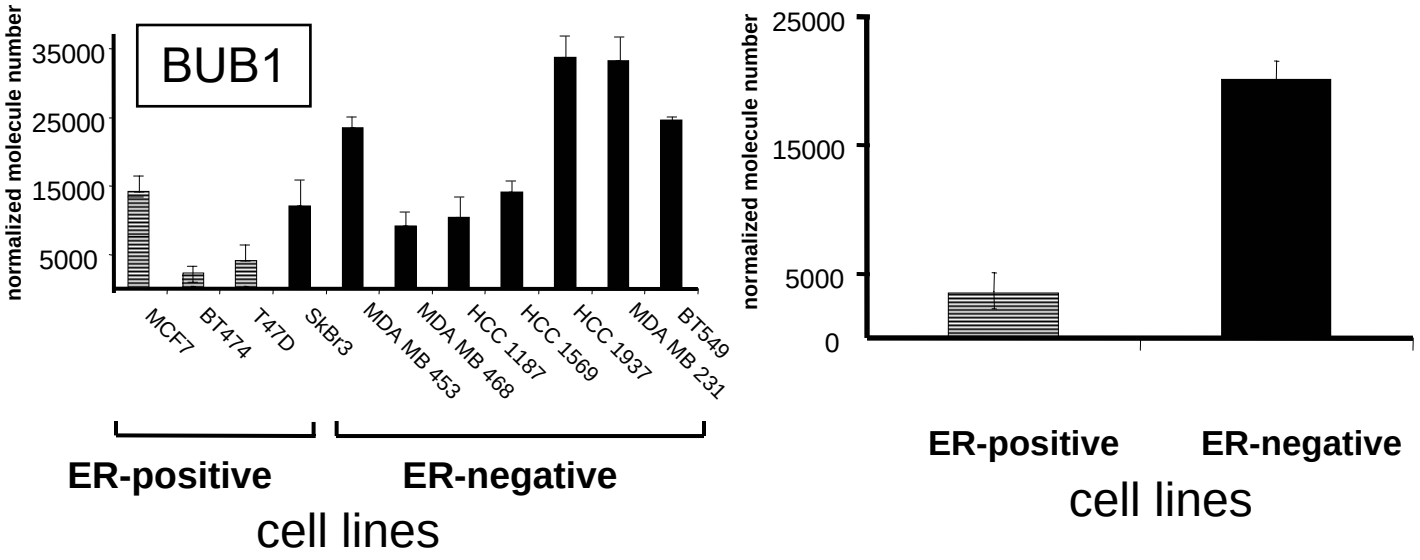
Cluster of kinases elevated in ER- negative, PR-negative, Her2/neu positive breast cancer

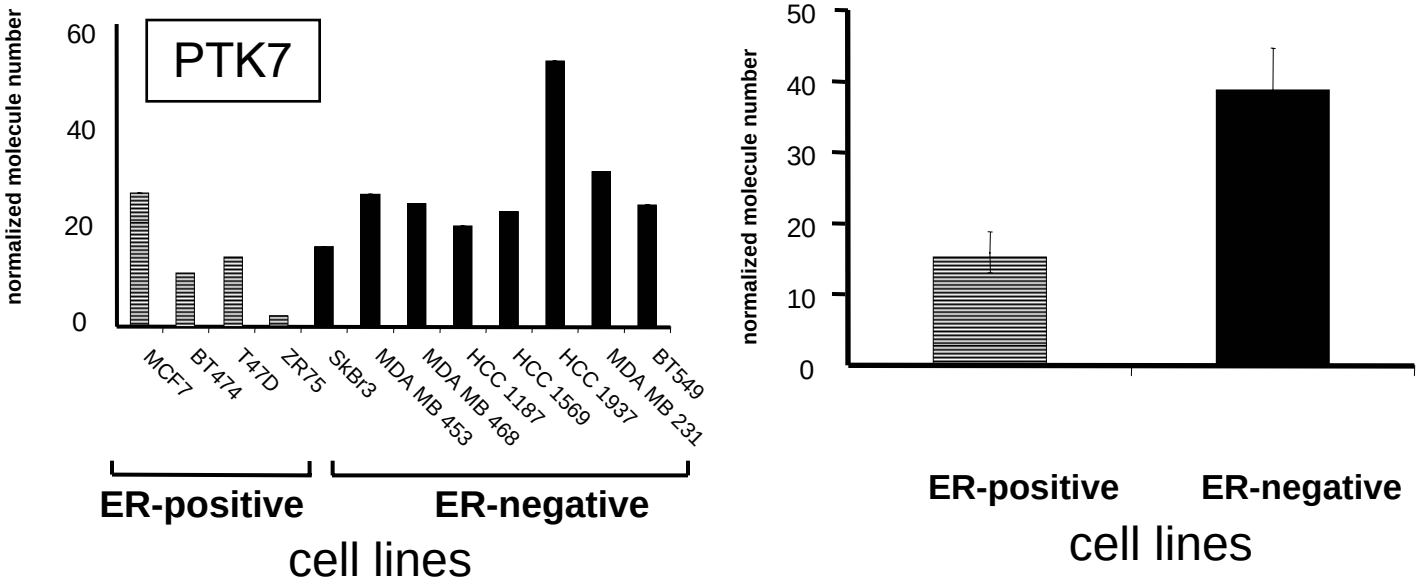
Second cluster of kinases elevated in ER-negative, PR-negative, Her2/neu positive breast cancer

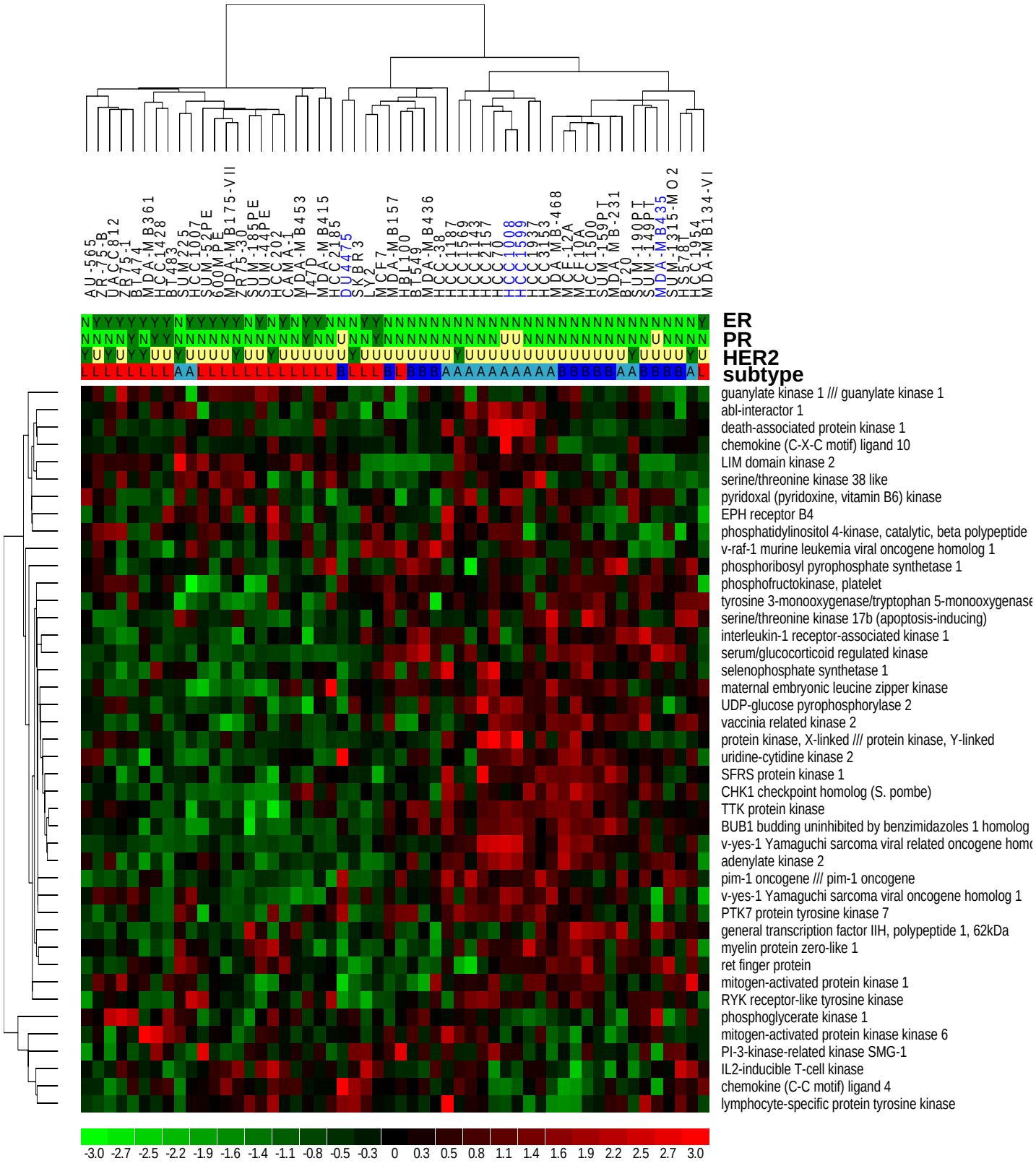
A


Set 1 tumors
70 kinases
Set 2 tumors
84 kinases
33 kinases
37 kinases
47 kinases


CHEK1
normalized molecule number
18000
14000
10000
6000
2000
MCF7
BT474
T47D
ZR75
SkBr3
MDA MB 453
MDA MB 468
HCC 1187
HCC 1569
HCC 1937
MDA MB 231
BT549
ER-positive
ER-negative
cell lines
normalized molecule number
16000
12000
8000
4000
0
ER-positive
ER-negative
cell lines

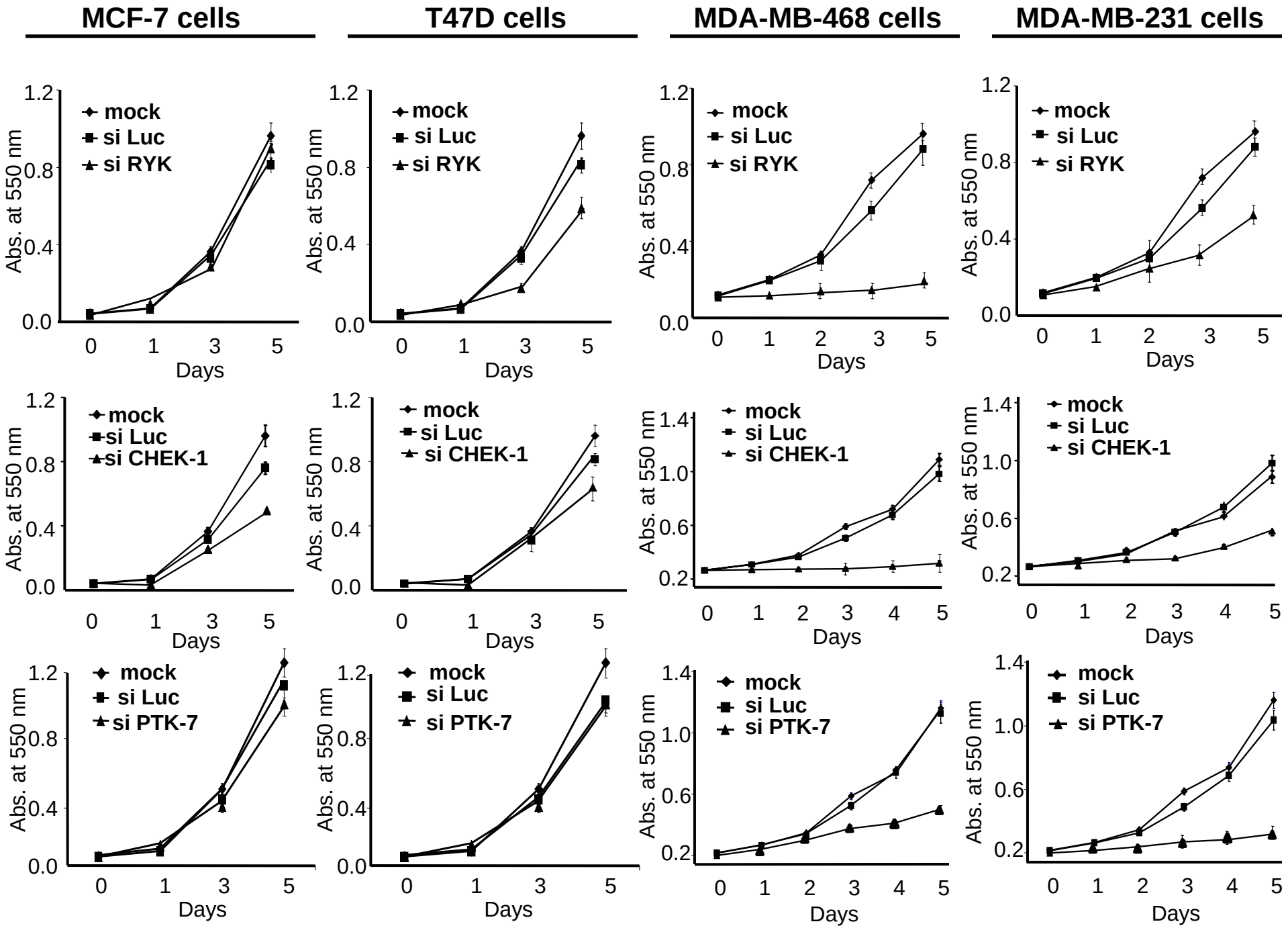

BUB1
normalized molecule number
35000
25000
15000
5000
MCF7
BT474
T47D
SkBr3
MDA MB 453
MDA MB 468
HCC 1187
HCC 1569
HCC 1937
MDA MB 231
BT549
ER-positive
ER-negative
cell lines
normalized molecule number
25000
15000
5000
0
ER-positive
ER-negative
cell lines


PTK7
normalized molecule number
60
40
20
0
MCF7
BT474
T47D
ZR75
SkBr3
MDA MB 453
MDA MB 468
HCC 1187
HCC 1569
HCC 1937
MDA MB 231
BT549
ER-positive
ER-negative
cell lines
normalized molecule number
50
40
30
20
10
0
ER-positive
ER-negative
cell lines


ER
PR
HER2
subtype
guanylate kinase 1 /// guanylate kinase 1
abl-interactor 1
death-associated protein kinase 1
chemokine (C-X-C motif) ligand 10
LIM domain kinase 2
serine/threonine kinase 38 like
pyridoxal (pyridoxine, vitamin B6) kinase
EPH receptor B4
phosphatidylinositol 4-kinase, catalytic, beta polypeptide
v-raf-1 murine leukemia viral oncogene homolog 1
phosphoribosyl pyrophosphate synthetase 1
phosphofructokinase, platelet
tyrosine 3-monooxygenase/tryptophan 5-monooxygenase
serine/threonine kinase 17b (apoptosis-inducing)
interleukin-1 receptor-associated kinase 1
serum/glucocorticoid regulated kinase
selenophosphate synthetase 1
maternal embryonic leucine zipper kinase
UDP-glucose pyrophosphorylase 2
vaccinia related kinase 2
protein kinase, X-linked /// protein kinase, Y-linked
uridine-cytidine kinase 2
SFRS protein kinase 1
CHK1 checkpoint homolog (S. pombe)
TTK protein kinase
BUB1 budding uninhibited by benzimidazoles 1 homolog
v-yes-1 Yamaguchi sarcoma viral related oncogene homo
adenylate kinase 2
pim-1 oncogene /// pim-1 oncogene
v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1
PTK7 protein tyrosine kinase 7
general transcription factor IIH, polypeptide 1, 62kDa
myelin protein zero-like 1
ret finger protein
mitogen-activated protein kinase 1
RYK receptor-like tyrosine kinase
phosphoglycerate kinase 1
mitogen-activated protein kinase kinase 6
PI-3-kinase-related kinase SMG-1
IL2-inducible T-cell kinase
chemokine (C-C motif) ligand 4
lymphocyte-specific protein tyrosine kinase
-3.0 -2.7 -2.5 -2.2 -1.9 -1.6 -1.4 -1.1 -0.8 -0.5 -0.3 0 0.3 0.5 0.8 1.1 1.4 1.6 1.9 2.2 2.5 2.7 3.0

Intersection of kinases identified in analysis

Cell cycle/DNA damage checkpoint	Gene bank accession number
BUB1	NM_001211
CHK1 checkpoint homolog	NM_001274
TTK protein kinase	NM_003318
serum/glucocorticoid regulated kinase	NM_005627
SFRS protein kinase 1	NM_003137
maternal embryonic leucine zipper kinase	NM_014791
Positive regulation of cell proliferation	
v-yes-1 Yamaguchi sarcoma viral related oncogene homolog	NM_005433
RYK receptor-like tyrosine kinase	NM_001005861
abl-interactor 1	NM_005470
chemokine (C-X-C motif) ligand 10	NM_001565
EPH receptor B4	NM_004444
serine/threonine kinase 38 like (NDR2)	NM_015000
v-raf-1 murine leukemia viral oncogene homolog	NM_002880
Receptor mediated endocytosis	
PI4-kinase	NM_058004
mindbomb homolog 1 (14-3-3)	NM_020774
Anti-Apoptosis	
vaccinia related kinase	NM_006296
death-associated protein kinase 1	NM_004938
mucosa associated lymphoid tissue lymphoma translocation gene 1	NM_006785
mitogen-activated protein kinase 1 (ERK2)	NM_002745
pim-1 oncogene	NM_002648
serine/threonine kinase 17b	NM_004226
v-raf-1 murine leukemia viral oncogene homolog	NM_002880
Positive regulation of transcription	
interleukin-1 receptor-associated kinase 1	NM_001569
protein kinase, X-linked	NM_005044
Cell adhesion/Cytoskeleton organization	
LIM domain kinase 2	NM_001031801
PTK7 protein tyrosine kinase 7	NM_002821
Immunoregulatory	
interleukin-1 receptor-associated kinase 1	NM_001569
lymphocyte-specific protein tyrosine kinase	NM_001042771
toll-like receptor 1	NM_003263
Cell-cell signaling	
chemokine (C-C motif) ligand 4	NM_002984
myelin protein zero-like 1	NM_003953
Metabolism	
phosphoglycerate kinase 1	NM_000291
phosphofructokinase, platelet	NM_002627
phosphoribosyl pyrophosphate synthetase 1	NM_002764
pyridoxal (pyridoxine, vitamin B6) kinase	NM_003681
selenophosphate synthetase 1	NM_004226
uridine-cytidine kinase 2	NM_012474
UDP-glucose pyrophosphorylase 2	NM_006759
adenylate kinase 2	NM_001625


MCF-7 cells
T47D cells
MDA-MB-468 cells
MDA-MB-231 cells
mock
si Luc
si RYK
si CHEK-1
si PTK-7
Abs. at 550 nm
Days

	ER-positive cell lines		ER-negative cell lines	
	MCF-7	T47D	MDA-MB-231	MDA-MB-468
ER-negative, Her2 negative mTOR pathway cluster				
EPHB4	slight inhibit.	no effect	slight inhibit.	inhibit.
serine/threonine kinase 38 like (NDR2)	no effect	no effect	no effect	no effect
death-associated protein kinase 1	no effect	no effect	inhibit	inhibit
pim-1 oncogene	no effect	no effect	no effect	no effect
LIM domain kinase 2	slight inhibit.	no effect	slight inhibit.	inhibit.
phosphoribosyl pyrophosphate synthetase 1	-	-	-	-
ER-negative, Her2 negative proliferation cluster				
BUB1	inhibit.	inhibit.	inhibit.	inhibit.
CHK1 checkpoint homolog	inhibit.	no effect	inhibit.	inhibit.
TTK protein kinase	inhibit.	inhibit.	inhibit.	inhibit.
Serum/glucocorticoid regulated kinase	-	-	-	-
SFRS protein kinase 1	no effect	no effect	-	no effect
maternal embryonic leucine zipper kinase	-	-	-	-
v-yes-1 Yamaguchi sarcoma v.r.o. homolog	slight inhibit.	slight inhibit	slight inhibit.	inhibit.
RYK receptor-like tyrosine kinase	no effect	no effect	inhibit.	inhibit.
phosphoglycerate kinase 1	-	-	-	-
selenophosphate synthetase 1	-	-	-	-
uridine-cytidine kinase 2	no effect	no effect	inhibit	inhibit
UDP-glucose pyrophosphorylase 2	-	-	-	-
adenylate kinase 2	-	-	-	-
vaccinia related kinase	no effect	no effect	no effect	inhibit.
ER-negative, Her2 positive paracrine signaling cluster				
v-raf-1 murine leukemia viral oncogene homolog	no effect	inhibit.	no effect	inhibit.
PTK7 protein tyrosine kinase 7	no effect	no effect	inhibit.	inhibit.
myelin protein zero-like 1	enhance growth	enhance growth	enhance growth	enhance growth
protein kinase, X linked	-	no effect	-	no effect
ER-negative., Her2 positive immunomodulatory cluster				
chemokine (C-X-C motif) ligand 10	no effect	no effect	-	slight inhibit.
interleukin-1 receptor-associated kinase 1	inhibit.	inhibit.	inhibit.	inhibit.
lymphocyte-specific protein tyrosine kinase	-	-	-	-
toll-like receptor 1	no effect	no effect	no effect	no effect
chemokine (C-C motif) ligand 4	inhibit.	inhibit.	inhibit.	inhibit.
pyridoxal (pyridoxine, vitamin B6) kinase	-	-	-	-
serine/threonine kinase 17b	-	-	-	-